JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 448–451 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.448–451.2005

PCR Amplification with Primers Based on IS*2404* and GC-Rich Repeated Sequence Reveals Polymorphism in *Mycobacterium ulcerans*

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Received 8 June 2004/Returned for modification 28 July 2004/Accepted 15 September 2004

We describe a simple genotyping method for *Mycobacterium ulcerans* based on PCR amplification of genomic regions between IS*2404* and a frequently repeated GC-rich sequence. Application of this method to a global collection produced 10 *M. ulcerans* genotypes corresponding to their geographic origin.

Mycobacterium ulcerans is the causative agent of Buruli ulcer (BU), an emerging disease characterized by chronic and necrotizing skin ulcers. BU occurs mostly in rural communities in tropical regions and is the third most common mycobacterial disease in humans after tuberculosis and leprosy (1).

Presently, the mode of transmission and other key aspects of the epidemiology of BU are not fully understood, partly due to the apparent lack of genetic diversity in *M. ulcerans* (1–3, 6, 8–10). New typing tools with high resolution need to be explored for the study and better understanding of the epidemiology of BU disease. In this study, we exploited the high copy number, species specificity, and potential of the insertion sequence IS*2404* to mediate genome rearrangements (8) and a frequently repeated GC-rich sequence (5′-CGG-CGG-CAA-CGG-CGG-CA-3′) in mycobacteria (7) to develop a simple PCR-based genotyping method for *M. ulcerans*.

The method, designated IS*2404*-Mtb2 PCR, involves the use of previously described outward-directed, IS*2404*-specific primers (MU3, 5′-CGC-GTG-GGT-CCC-TCG-GGT-CT-3′; and MU4, 5′-ATC-GCC-GAA-GCC-TGC-CGG-GAT-3′) (8) in combination with the oligonucleotide Mtb2 (5′-CGG-CGG-CAA-CGG-CGG-C-3′) targeting the repeated GC-rich motif to amplify DNA sequences located between adjacent and appropriately oriented copies of these elements. Previously, we used Mtb2 in combination with primers IS1 and IS2, directed at inverted repeats flanking IS*6110*, to develop a PCR-based epidemiological tool for the differentiation of *Mycobacterium tuberculosis* isolates (5).

We confirmed the presence of Mtb2 sequences in *M. ulcerans* by BLAST search of the *M. ulcerans* sequence database available on the BURULIST web server of the Institute Pasteur de Paris, Paris, France (http://genopole.pasteur.fr/Mulc/List.html).

Strains exhibiting differences in their genome architecture with respect to these elements show different banding patterns and are consequently differentiated. The *M. ulcerans* typing method 2426 PCR developed by Stinear et al. is based on this principle but amplifies regions between two insertion elements, IS*2404* and IS*2606* (9).

To investigate the IS*2404*-Mtb2 PCR, whole genomic DNA was prepared by the standardized method described by van Embden and coworkers (11). Briefly, suspensions of bacteria in Tris-EDTA buffer (pH 8.0) were digested with lysozyme (1 mg/ml). Suspensions were then treated with proteinase K (0.1 mg/ml) and sodium dodecyl sulfate (to 1%) and incubated with NaCl (0.6 M) and *N*-acetyl-*N*,*N*,*N*-trimethyl ammonium bromide. DNA was extracted with chloroform-isoamyl alcohol and precipitated with isopropanol. For the PCR amplification, each reaction mixture contained 3 μl of genomic DNA (about 10 ng/μl), 12.5 μl of water, 3 μl of PCR buffer with 1.5 mM $MgCl_2$, a 20-pmol concentration of each primer, 5 mM deoxynucleotide triphosphate, 6 μl of Q solution, and 1 U of HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany) in a final volume of 30 μl.

PCRs were run on a PTC 100 thermocycler (MJ Research, Waltham, Mass.) and consisted of an initial *Taq* activation and denaturation step of 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. The Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany) was used to electrophoretically separate 1 μl of PCR product. The resulting banding profiles were analyzed with the Bionumerics version 3.0 computer software (Sint-Martens-Latem, Belgium).

We tested this method with 32 *M. ulcerans* isolates representing a geographically diverse collection and 1 isolate (ITM 00–1026) classified as *Mycobacterium marinum* (2) but also harboring the *M. ulcerans* specific IS*2404* element (4) and described previously as a "missing link" (4) (Table 1).

All the *M. ulcerans* isolates with the exception of isolate ITM 00–1441 were obtained from humans. Isolate ITM 00–1441 was recovered from an aquatic insect (Gerris sp.) in Benin (Portaels et al., unpublished results).

Comparison of IS*2404*-Mtb2 banding profiles revealed 10 different patterns, corresponding to the geographic origin of the isolates (Fig. 1). All of the 16 isolates originating from six different African countries produced an identical profile. The environmental isolate ITM 00–1441 also had the same profile

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TABLE 1. *M. ulcerans* strains investigated in this study

Isolate	Origin[a]	Source (other strain designation)[b]	2426 PCR type[c]	IS*2404*-Mtb2 type
ITM 5142	Australia	ATCC 19423	Victorian	Victorian
ITM 94-1326	Australia	L.S., 93160339	Victorian	Victorian
ITM 94-1329	Australia	F.P., 144727	Victorian	Victorian
ITM 9550	Australia	D.D., 17679	NT	Victorian
ITM 9540	Australia	D.D., 11098/70	NT	Queensland
ITM 94-1324	Australia	F.P., 176862	NT	Queensland
ITM 9537	PNG	K.J., 11878/70	PNG I	PNG I
ITM 94-1331	PNG	K.J., 186395	PNG II	PNG II
ITM 03-524	PNG	F.P.	NT	PNG I
ITM 94-1328	Malaysia	K.J., 186510	Malaysian	Malaysian
ITM 7922	French Guyana	V.V., 141090018	NT	South American
ITM 842	Surinam	V.K., 701357	Surinam	South American
ITM 5114	Mexico	P.L.	Mexican	Mexican
ITM 5143	Mexico	P.L.	Mexican	Mexican
ITM 8756	Japan	ATCC 33728	Asian	Japanese
ITM 98-912	China	F.P.	Asian	Chinese
ITM 5150	D.R. Congo	F.P.	NT	African
ITM 5152	D.R. Congo	F.P.	African	African
ITM 5155	D.R. Congo	F.P.	African	African
ITM 94-662	Ivory Coast	F.P.	NT	African
ITM 96-657	Angola	F.P.	NT	African
ITM 96-658	Angola	F.P.	African	African
ITM 97-321	Ghana	F.P.	NT	African
ITM 97-483	Ghana	F.P.	NT	African
ITM 97-610	Ghana	F.P.	African	African
ITM 94-856	Benin	F.P.	African	African
ITM 97-111	Benin	F.P.	African	African
ITM 98-239	Benin	F.P.	NT	African
ITM 00-0040	Benin	F.P.	NT	African
ITM 00-1213	Benin	F.P.	NT	African
ITM 00-1441	Benin	F.P.	NT	African
ITM 02-279	Cameroon	F.P.	NT	African
ITM 00-1026	France	F.P.	NT	*M. marinum*[d]

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[c] Genotype designation as determined by 2426 PCR (9). NT, not tested.
[d] *M. marinum* genotype designation as determined by amplified fragment length polymorphism and phenotypic tests (4).

as that of isolates from human origin. This homogeneous pattern is designated the African genotype. Two different genotypes were identified in east Asia (Japan and China genotypes), Australia (Victorian and Queensland genotypes corresponding to southeastern and northern Australia, respectively) and in Papua New Guinea (PNG I and II genotypes). The Malaysian genotype was nearly identical to the Queensland genotype, differing by the absence of one band in its profile. Similarly, there was one band difference between the profiles of the PNG I and Victorian genotypes. A South American genotype comprising identical profiles of two isolates from Surinam and French Guyana was shown to be different from the unique profiles of the Mexican and the missing link genotypes.

A dendrogram comparing the relationship among the genotypes was constructed with the similarity scores of the banding profiles and shows clustering of isolates from the same geographic region (Fig. 2). Among the geographic groupings, the Southeast Asian cluster (comprising the Malaysian, PNG I and II, Victorian, and Queensland genotypes) and the African genotype display about 68% similarity and represent the most closely related *M. ulcerans* genotypes originating from different geographic regions. The Mexican genotype and the missing link constituted a separate cluster least related to the other *M. ulcerans* genotypes. The link between these two genotypes may be consistent with the observation that both isolates contain comparatively fewer IS*2404* elements (3).

Overall, the results of the IS*2404*-Mtb2 PCR method corroborate previous *M. ulcerans* typing data (2, 3, 6, 8, 9, 10) in revealing a clonal population structure in *M. ulcerans* within a given geographic region. More interestingly, both 2426 PCR (9) and IS*2404*-Mtb2 PCR revealed two different *M. ulcerans* genotypes in Australia and Papua New Guinea.

2426 PCR analysis of a set of overlapping isolates used in this study produced nine *M. ulcerans* genotypes and has until now been the most discriminatory method for *M. ulcerans*. However, using the IS*2404*-Mtb2 PCR method, we could for the first time identify two different genotypes (i.e., Chinese and Japanese) in east Asia and have consequently been able to resolve 10 different *M. ulcerans* genotypes globally. In addition to the increased resolution, this method is reproducible and technically easy to perform. The results of this work allow for

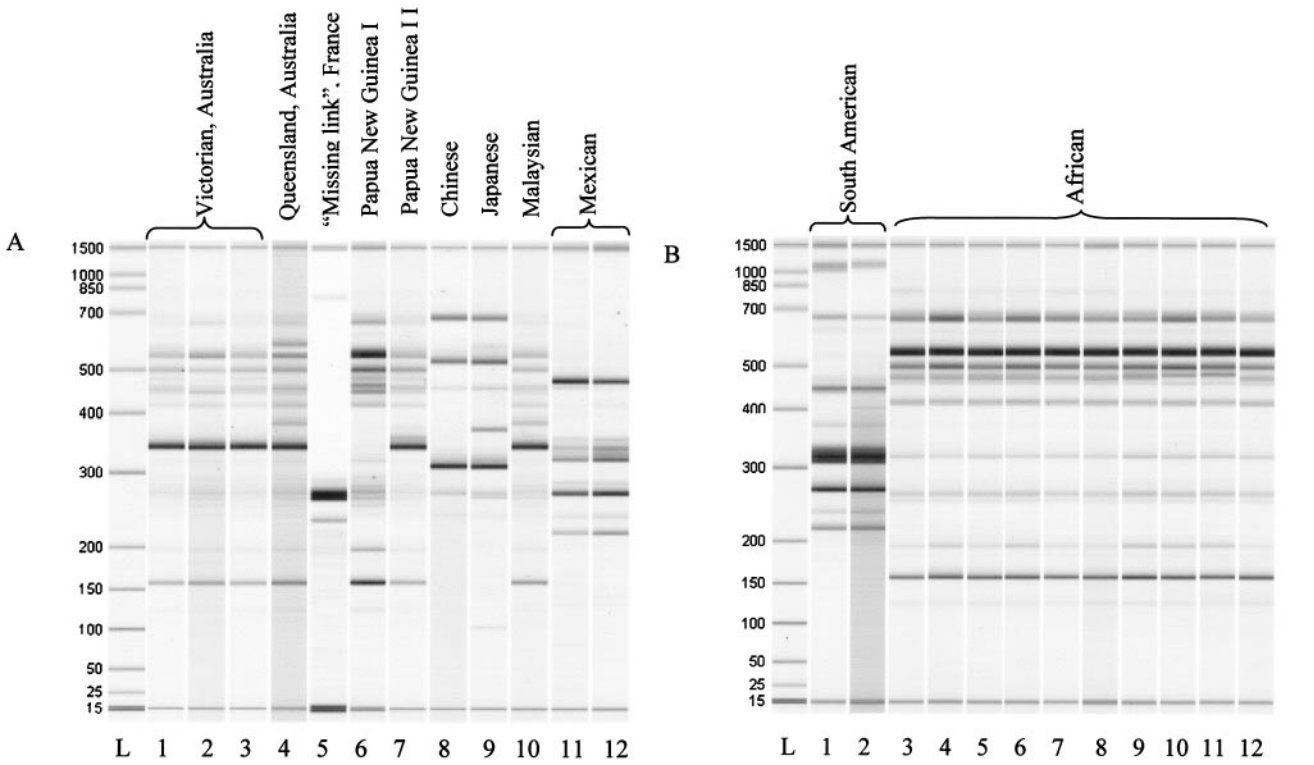

FIG. 1. IS*2404*-Mtb2 PCR analysis of *M. ulcerans* isolates from different geographic regions and isolate ITM 00–1026, the missing link. (A) Lanes: L, DNA ladder; 1, ITM 5142; 2, ITM 1326; 3, ITM 1329; 4, ITM 9540; 5, ITM 00–1026; 6, ITM 9537; 7, ITM 94–1331; 8, ITM 98–912; 9, ITM 8756; 10, ITM 94–1328; 11, ITM 5143; 12, ITM 5114. (B) Lanes: L, DNA ladder; 1, ITM 842; 2, ITM 7922; 3, ITM 5150; 4, ITM 5155; 5, ITM 96–658; 6, ITM 96–657; 7, ITM 97–610; 8, ITM 02–279; 9, ITM 00–0040; 10, ITM 00–1213; 11, ITM 00–1441; 12, ITM 94–662. The 15- and 1,500-bp bands in all lanes represent lower and upper internal markers.

the speculation that limited IS*2404*-mediated genome reshuffling may be responsible for the apparent lack of genotype diversity in *M. ulcerans*, especially among African isolates. Variation in intensities of bands that may lead to inaccuracies in the assignment of genotypes could be a limitation of this approach. Here, this concern was addressed by using approximately equal concentrations of template DNA and maintaining uniform amplification conditions. High annealing temperature (65°C) was also used to ensure specific binding of primers to target sequences. While IS*2404*-Mtb2 PCR, like previous *M. ulcerans* genotyping methods, may not provide the requisite level of discrimination necessary for local epidemiological investigations, it could, however, be considered as a simple and rapid means of differentiating among *M. ulcerans* from different geographic regions.

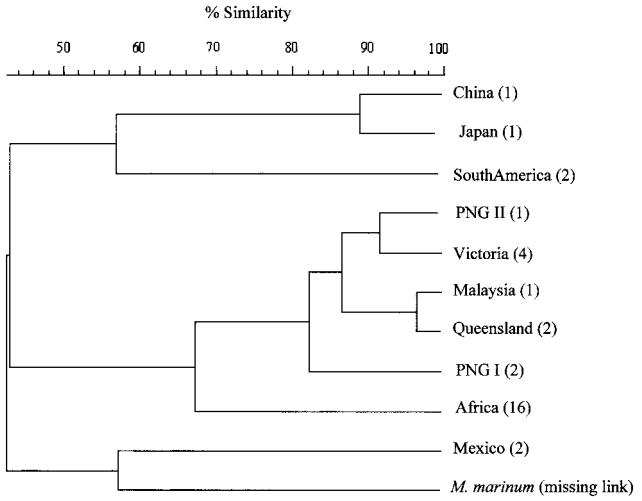

FIG. 2. Dendrogram showing relationships among the 33 isolates. Cluster analysis was performed by the unweighted pair group method, using the arithmetic average algorithm with the Bionumerics version 3.0 computer software (Sint-Martens-Latem, Belgium). Percent similarity was calculated with the band-based Dice correlation coefficient. The numbers in parentheses represent the number of isolates tested.

This work was partly supported by a grant from the Fund for Scientific Research, Flanders (Belgium) (F.W.O-Vlaanderen. contract no. G.0301.01). A.A was supported by a grant from the Damien Foundation (Brussels, Belgium).

We are grateful to Juan Carlos Palomino, Karim Chemlal, Leen Rigouts, Anandi Martin, and Pieter Stragier for assistance and advice. We also thank Pim de Rijk, Krista Fissette, and Æecile Uwizeye for excellent technical work.

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